# THE STRUCTURE AND METABOLISM OF A CRUSTACEAN INTEGUMENTARY TISSUE DURING A MOLT CYCLE[1]

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"And the body form is moulded by the epidermis. It is the epidermis which shapes the organism in all its details; the other tissues, which support and nourish and connect one part with another, follow the lead which the epidermis gives. Even the great integrating systems, the endocrine organs and the central nervous system, are historically a part of the ectoderm, and where they influence the body form they do so chiefly by the activation of the epidermis."

In these few sentences Wigglesworth (1945, p. 23) outlined one of the great challenges of arthropod physiology. It is known that during the period preceding ecdysis the arthropod epidermis undergoes profound changes in structure which probably reflect the synthesis of a new exocuticle (or exoskeleton) to encompass the reshaped and enlarged animal (Kuhn and Piepho, 1938; Travis, 1955, 1958; Wigglesworth, 1933). The preparation for molting is also accompanied by a 50 to 1900% increase in oxygen consumption by the whole animal (Bliss, 1953; Edwards, 1950, 1953; Nyst, 1941; Poulson, 1935; Schneiderman, 1952; Schneiderman and Williams, 1953; Scudamore, 1947), which means that the metabolism of some or all of the tissues is vastly increased.

This paper describes the structure and metabolism of the integumentary tissue of the land crab, *Gecarcinus lateralis,* during the molt cycle. Integumentary tissue is comprised of two sheets of epidermal cells separated by a layer of connective tissue.

As a source of integumentary tissue, the branchiostegites, sheets of tissue which form the covering of the branchial chambers, were selected for several reasons. A small piece of tissue, 10 to 20 mm.$^2$, could be excised from each branchiostegite without affecting the length of the molt cycle. Routinely, two samples were taken from the same animal at different times in the molt cycle. At its maximum, the integumentary tissue of the branchiostegites is only 450 $\mu$ thick. Without being sliced it should, therefore, permit adequate diffusion of oxygen to interior cells (Field, 1948). The use of a tissue which did not have to be sliced reduced to a minimum changes in oxygen consumption due to loss of coenzymes by diffusion from injured cells or by the action of nucleotidases (Mann and Quastel, 1941).

Drach (1939) subdivided the crustacean molt cycle into stages A through D,

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depending on the state of exoskeleton. The exoskeleton was pliable in stages A and B, 4 to 8 days immediately following ecdysis, whereas stage C, the three-month intermolt period, was marked by a rigid exoskeleton. The 15- to 30-day premolt period, during which the two outer layers of the new exoskeleton were formed, was designated as stage D.

The cytological changes of the epidermis and other cells of the integumentary tissue of *Gecarcinus* were correlated with the exoskeletal changes described by Drach. Metabolic studies of the integumentary tissues were then undertaken. The rates of oxygen consumption of pieces of integumentary tissues taken from a series of animals in each stage of the molt cycle were measured. The exact stage of each animal was determined from inspection of sections of tissue removed from the animal on the day of the metabolic studies.

Limbs autotomized from crustaceans are regenerated during the premolt period (Bliss, 1956). In the present study, when the size of regenerating limb buds was correlated with the structure of the epidermis, it was found that regeneration of limbs is complete before any morphological changes are detected in the epidermis.

A report on some of these results has appeared previously (Skinner, 1958).

## Materials and Methods

### 1. *Selection and maintenance of animals*

Specimens of the land crab, *Gecarcinus lateralis*, collected in Bermuda or Bimini, were maintained in the laboratory as described by Bliss (1953). Animals ranging in carapace width from 3.5 to 5 cm. were used. At each feeding period (*i.e.*, every ten days), regenerating limb buds were measured. During the premolt period, they were measured more frequently.

### 2. *Removal of tissue*

Animals were anesthetized by chilling at 4° C. for 15 to 20 minutes. A piece of tissue approximately 3 mm. by 4 mm. was removed from one branchiostegite and the opening in the branchial chamber was covered by a piece of plastic sealed in place by melted paraffin. Operated animals were returned to their individual containers and observed until external signs of an approaching ecdysis were seen (*i.e.*, growth of regenerating limb buds, swelling of pericardial sacs (Bliss, 1953, 1956); depressibility of the exoskeleton (Drach, 1939)). A second piece of tissue was then removed from the other branchiostegite, the crab being similarly treated and observed until ecdysis. The time from tissue removal until ecdysis was thus known. These data, coupled with the histological condition of the tissue, permitted the determination of the duration of each stage of the premolt period.

### 3. *Histological and histochemical methods*

Pieces of tissue were fixed in Bouin's solution, dehydrated in ethanol, imbedded in paraffin and sectioned at 7 to 10 $\mu$. Sections were stained with either Mallory's triple stain or phosphotungstic acid.

RNA was visualized by staining with dilute solutions (0.01%) of methylene blue over a pH range of 3 to 6.2. In that pH range, most basic staining is

attributed to nucleic acids, since the carboxyl group of proteins has a pK of 2 and is not dissociated (Swift, 1955). Control sides were subjected to RNase[3] hydrolysis before methylene blue staining. For contrast, the sections were counterstained with dilute eosin.

The periodic acid-Schiff method was used to demonstrate the presence of glycogen. Control sections in this series were pretreated with salivary amylase. The tissues of 75 animals were studied.

4. *Preparation of tissues*

Pieces of tissue (50 to 100 mg. wet weight) were cut from the branchiostegites. During the intermolt period, the epidermis is tightly attached to the innermost region of the exoskeleton, the membranous layer (Fig. 1), which can be separated

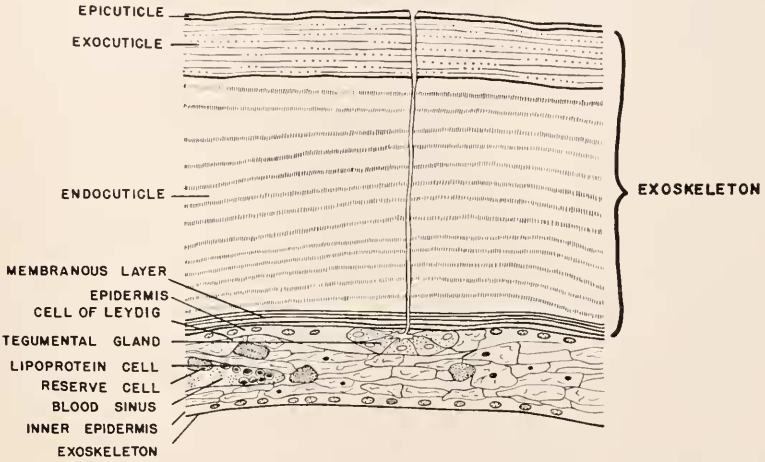


FIGURE 1. Diagrammatic cross-section of the integumentary tissue of an intermolt *Gcarcinus,* drawn to scale. The epidermal layer described is the one adjacent to the thick outer exoskeleton. 100 × magnification.

as a thin sheet from the outer region of the exoskeleton. To avoid disrupting the epidermis, intermolt tissues were removed with the membranous layer attached. During that part of the premolt period (stage $D_1$ and later) when the membranous layer is being resorbed, the remainder of the old exoskeleton can be lifted away from the integumentary tissues. Pieces of isolated tissue, with membranous layer (intermolt), without membranous layer (early premolt), or with newly synthesized exoskeleton (late premolt and early postmolt) were weighed on a Roller Smith torsion balance and immersed in 0.5 ml. iced *Carcinus* perfusion fluid. They were then blotted on filter paper and placed in the Warburg vessels.

5. *Oxygen consumption measurements*

The oxygen consumption of pieces of tissue was determined manometrically. The main chamber of five-ml. Warburg vessels received buffered (0.02 *M* Tris,

[3] The following abbreviations are used: RNase, ribonuclease; Tris, trishydroxymethylamino methane; PAS, periodic acid-Schiff; DNP, dinitrophenol.

pH 7.7) *Carcinus* perfusion fluid (Pantin, 1946) containing 15 $\gamma$ streptomycin and 4 $\gamma$ penicillin per ml. The center well contained 0.1 ml. of a 10% solution of potassium hydroxide. Dinitrophenol and Krebs substrates (Krebs, 1950), when added, were placed in the sidearm. To each 0.75 ml. was added 0.25 ml. of a solution containing the following substrates in milliequivalents/liter: 4.9 pyruvate, 4.9 glutamate, 5.4 fumarate, 9.2 glucose. In experiments testing the effect of cyanide, potassium hydroxide was replaced by 0.1 ml. of a calcium

TABLE I

*Schedule of premolt and early postmolt events in* Gecarcinus

| Stage | Initiation (days before ecdysis) | Completion (days before ecdysis) | Event |
|---|---|---|---|
| $D_0$ | 25+ | ? | Gastrolith formation |
| $D_0$ | 25 | 5 to 10 | Regeneration of autotomized limbs |
| $D_1$ | 12 | 10 | Resorption of old exoskeleton, beginning with the membranous layer; increase in height of epidermal cells to 10 $\mu$. |
| $D_1$ | 10 | 8 | Further enlargement of epidermal cells to 30 $\mu$, separation from old exoskeleton by resorption of membranous layer. |
| $D_2$ (early) | 7 | 5 | Formation of two-layered epicuticle |
| $D_2$ (late) | 4 | 2 | Formation of exocuticle |
| $D_3$ | 1 | 0.5 | Slight decrease in size of epidermal cells |
| $D_4$ | 0.5 | 0 | Blood pink |
| | | Ecdysis | |
| A | 0 | 1 | Epidermal cells shrink slightly |
| B | 1 | 5 | Formation of endocuticle, about 7 $\mu$ each day |
| $C_1$ and $C_2$ | 5 | ? | Formation of endocuticle continued, at the same rate |

hydroxide-potassium cyanide suspension, of the concentration required to saturate the gas phase at the desired molarity (Robbie, 1948). Calcium hydroxide was used as the alkali in control vessels.

The total volume, including the tissue, was 1 ml. The flasks were incubated at 25° C. and shaken at the rate of 130 oscillations per minute.

At the end of the experiment, tissues were rinsed in distilled water, blotted on filter paper and dried in a 100° oven for 24 hours. They were then weighed on a Sartorius balance. The rate of oxygen consumption ($Q_{O_2}$) was expressed as $\mu$l. $O_2$/mg. dry weight/hour. At least two aliquots of tissue were taken from each animal.

## Results

### 1. *The molt cycle*

The duration of the molt cycle of a mature *Gecarcinus lateralis* (carapace width 3 cm. or greater) is four to six months (Table I). The intermolt period, $C_1$ to $C_4$, comprises all of the cycle except for a 30-day premolt period ($D_0$ through $D_4$) and a short postmolt period (A through B) when synthesis of the exoskeleton continues. In the premolt period, animals regenerate autotomized limbs, resorb more than three-fourths of the old exoskeleton and synthesize an exoskeleton to replace the one lost at ecdysis.

During the premolt period, the weight of animals increased by 13 to 30% of the intermolt value, due to the absorption of water. After ecydysis, animals weighed one-half as much as during the preceding intermolt period. Within 10 days after ecdysis, they had regained the weight lost at ecdysis and an additional increment due to growth, which occurs only during the early postmolt period when the exoskeleton is still pliable. After each ecdysis, there was a 1 to 7% increase in carapace width and a 6 to 22% increase in weight.

### 2. *Cytology of the integumentary tissue*

The branchiostegites are bounded on their inner and outer surfaces by single sheets of epidermis (Fig. 1). The epidermal layer bounding the inner surface of the branchiostegites synthesizes a 7-$\mu$-thick layer of cuticle with staining characteristics similar to those of the two-layered epicuticle. The outer epidermal layer, on the other hand, synthesizes the thick exoskeleton, composed of a 7-$\mu$ epicuticle, a 30-$\mu$ exocuticle and a 200- to 400-$\mu$ endocuticle. Both epicuticle and exocuticle are synthesized during the premolt period, while the endocuticle is formed during the postmolt period. In this study, attention has been directed to the structural changes of the outer epidermal layer.

Between the two epidermal layers there is a layer of connective tissue, the bulk of which is composed of cells of Leydig (Cuénot, 1893). Among the cells of Leydig are scattered reserve cells (Hardy, 1892) and small blood sinuses which contain lipoprotein cells (Sewell, 1955). At the inner edge of each epidermal layer there are tegumental glands whose secretory cycle is not correlated with the molt cycle, since both replete and empty glands are present at all stages of the molt cycle.

### 3. *Cytological changes of the integumentary tissue*

#### a. Epidermis

Integumentary tissue removed from an animal 16 days before ecdysis (Fig. 2) is identical to that from an intermolt animal (Fig. 1). Resorption of the membranous layer, the innermost region of the exoskeleton, begins approximately 11 days before ecdysis, and the nuclei of the epidermal cells have enlarged (Fig. 3). As the membranous layer is digested, its staining characteristics change. Intact membranous layer is PAS-negative while partially digested membranous layer is PAS-positive, indicating that a material with adjacent hydroxyl groups is made

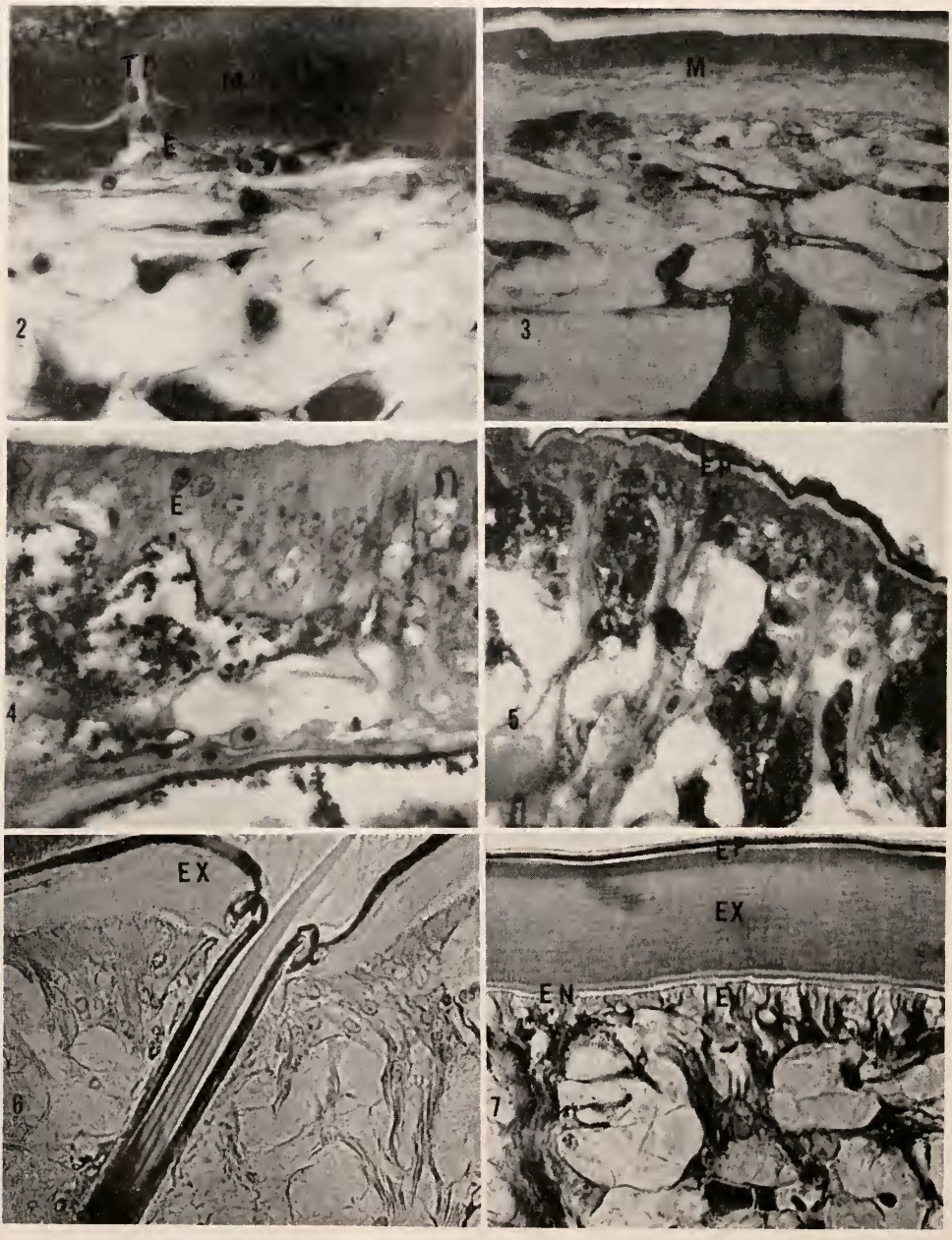


FIGURE 2, Stage $D_0$. Integumentary tissue from an animal 16 days before ecdysis. Only the nuclei of the epidermal cells (E) are visible beneath the membranous layer (M) of the exoskeleton. The duct of a tegumental gland (TD) can be seen entering the exoskeleton. Note that the tissue is identical to tissue from an intermolt animal (Fig. 1).

FIGURE 3, Stage $D_1$. Integumentary tissue from an animal 11 days before ecdysis. The membranous layer (M) is being resorbed.

available for oxidation by $HIO_4$ and for consequent reaction with the leucho-fuchsin dye. The nature of the reactive material is unknown. However, it is known that the crustacean exoskeleton is composed of approximately equal amounts of chitin, which is PAS-negative, and protein (Lafon, 1948). Part of the protein may be a mucoprotein with a carbohydrate component possessing adjacent hydroxyl groups.

Eight days before ecdysis there is complete separation of the exoskeleton from the epidermal cells which have enlarged further (Fig. 4). Synthesis of both layers of the epicuticle has been completed by the fifth day preceding ecdysis (Fig. 5), and the 30-$\mu$-thick exocuticle is formed during the following two days (Fig. 6). The endocuticle, whose formation begins on the second day following ecdysis, is thickened at the rate of 7 $\mu$ per day (Fig. 7) for at least a week (the period of time during which samples of tissue were taken).

b. Other integumentary cells

Near the end of the intermolt period, the number of lipoprotein cells increases. The cytoplasm of these cells becomes dotted with acidophilic granules during the early premolt stages. As ecdysis approaches, the cells increase their granular contents and move to the epidermis. By the time the epicuticle has been completed, near the end of the premolt period, the lipoprotein cells have disappeared. Their disappearance, coupled with the similar staining characteristics of the outer epicuticle and the lipoprotein cell granules, leads to the speculation that the granules are incorporated into the epicuticle. The changes of the lipoprotein cells of *Gecarcinus* parallel those of the homologous cells of the green crab (Sewell, 1955) to this point. However, in the green crab the small granules coalesce to form one large droplet immediately preceding ecdysis. In *Gecarcinus*, the granules do not coalesce; rather, large cells with homogeneous cytoplasm, similar to the reserve cells described by Hardy (1892), are seen at all stages of the molt cycle.

c. Glycogen metabolism of the integumentary tissue

As can be seen in Table II, the glycogen content of the outer epidermal layer changes markedly during the premolt period. As the old exoskeleton is broken down and new exoskeleton synthesized, there is an increase in glycogen content of the epidermis. The glycogen content of the cells of Leydig also increases before and decreases after ecdysis, suggesting that these cells serve as intermediates in glycogen metabolism, probably receiving glucose from the blood and releasing it to the epidermis.

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FIGURE 4, Stage $D_1$. Integumentary tissue from an animal 8 days before ecdysis. The epidermal cells (E) are greatly enlarged and are completely separated from the old exoskeleton.

FIGURE 5, Stage $D_2$ (early). Integumentary tissue from an animal 5 days before ecdysis. The epidermal cells (E) have completed synthesis of both layers of the epicuticle (EP).

FIGURE 6, Stage $D_2$ (late). Two-layered epicuticle completed; exocuticle (EX) partially formed. Hair follicle visible.

FIGURE 7, Stage B. Integumentary tissue from an animal two days after ecdysis. Epicuticle, exocuticle as above. First layers of endocuticle (EN) seen. Epidermal cells have decreased in size and their nuclei are no longer visible.

TABLE II

*Glycogen content of cells of the integumentary tissue*

| Stage | Epidermal cells | | | Cells of Leydig | Lipoprotein cells | |
|---|---|---|---|---|---|---|
| | Size | | Glycogen content | Glycogen content | Number | Contents |
| | Height, $\mu$ | Width, $\mu$ | | | | |
| Intermolt $C_4$ | 4 | 10–17 | + | + | ++++ | − − − |
| Premolt $D_0$ | 4 | 10–17 | + | + | +++++ | |
| Premolt $D_1$ | 10 | 10–17 | + | ++ | +++ | + |
| Premolt $D_2$ (early) | 30 | 10–17 | ++ | +++ | ++ | ++ |
| Premolt $D_2$ (late) | 100 | 10–17 | +++ | +++ | + | +++ |
| Premolt $D_3$ | 80 | 10–17 | ++ | +++ | + | ++ |
| Premolt $D_4$ | 80 | 10–17 | + | ++ | + | ++ |
| Postmolt A | 20 | 10 | + | + | − | |
| Postmolt B | 10 | 10 | + | + | − | |

4. *Formation of gastroliths*

*Gecarcinus* stores calcium resorbed from the old exoskeleton as concretions (gastroliths) which form in the lining of the stomach. In *Gecarcinus*, gastrolith formation begins about 30 days before ecdysis. Within three days after ecdysis, the gastroliths have disappeared completely.

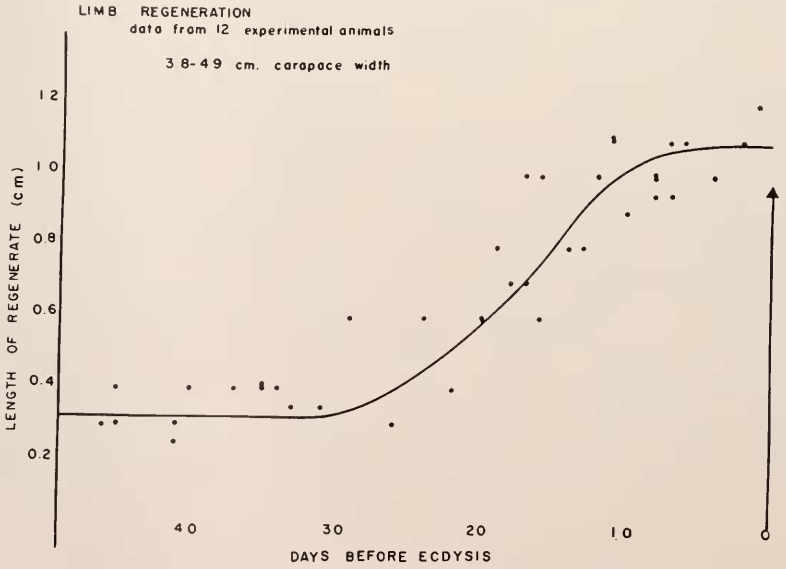


FIGURE 8. Compilation of growth curves of regenerating limbs of 12 *Gecarcinus*. Note plateau until 25 days before ecdysis, when limb buds begin to grow again. Limb bud reaches maximum size approximately 10 days before ecdysis.

5. *Regeneration of limbs*

Within the first two to three weeks after a limb is autotomized, a small limb bud, 2–4 mm. long, grows out from the scar tissue which forms over the stump of the autotomized limb. The limb bud remains in this form until the succeeding premolt period when it resumes growth (Bliss, 1956).

In Figure 8, the length of regenerating limbs of animals used in this study is plotted against time. It can be seen that about 30 days before molt, limb buds begin to elongate, that they grow at a rapid rate for approximately 20 days, completing their growth about 10 days before ecdysis.

6. *Oxygen consumption of the integumentary tissue*

a. Rate of oxygen consumption at each stage of the molt cycle

The integumentary tissues of intermolt, early premolt (stages $D_1$, $D_{2\ early}$) and early postmolt (stage B) consume oxygen at approximately equal rates (Table III; Fig. 9). The $Q_{O_2}$ of integumentary tissue synthesizing the 30-$\mu$-thick

TABLE III

*The mean $Q_{O_2}$ of the integumentary tissue at each stage of the molt cycle*

| Stage | Number of animals | Mean $Q_{O_2}$ | Standard deviation |
|---|---|---|---|
| $C_4$ | 8 | 0.53 | 0.14 |
| $D_0$ | 5 | 0.30 | 0.14 |
| $D_1$ | 7 | 0.49 | 0.19 |
| $D_{2\ (early)}$ | 3 | 0.46 | — |
| $D_{2\ (late)}$ | 11 | 0.85 | 0.28 |
| A | 2 | 0.72 | — |
| B | 6 | 0.38 | 0.08 |

exocuticle ($D_{2\ late}$) is significantly higher than that of intermolt tissue (Table III; Fig. 9). The $Q_{O_2}$ of tissue removed from two animals immediately after ecdysis (stage A) is also significantly higher than that of intermolt tissue.

The mean $Q_{O_2}$ of tissues removed from animals in stages $D_1$, $D_{2\ early}$, and B has been tested statistically against the mean $Q_{O_2}$ of tissues removed from $C_4$ animals. They have been found not to differ significantly. However, the mean $Q_{O_2}$ of tissues removed from $D_0$ animals is significantly lower than that of the tissue from intermolt animals. No explanation can be given for this decrease in respiratory rate at the initiation of the premolt period. The mean $Q_{O_2}$ of tissues removed from animals in stage $D_{2\ late}$, when the exocuticle is being formed, is significantly higher than the mean $Q_{O_2}$ of tissues from animals at all other stages.

b. Effect of cyanide and dinitrophenol

Both $10^{-4}$ and $10^{-5}$ *M* cyanide inhibited oxygen consumption of integumentary tissues from intermolt and premolt animals by 60 to 95%. As seen in Figure 10, $10^{-4}$ and $10^{-5}$ *M* DNP increased the oxygen consumption of the integumentary tissues.

c. Effect of endogenous substrates in blood serum and of Krebs substrates

Tissues bathed in *Carcinus* perfusion fluid (Pantin, 1946), which was 25% (v/v) in *Gecarcinus* blood serum, respired at a greater rate than tissues bathed in the salt solution alone. The increase was in the order of 50 to 200%.

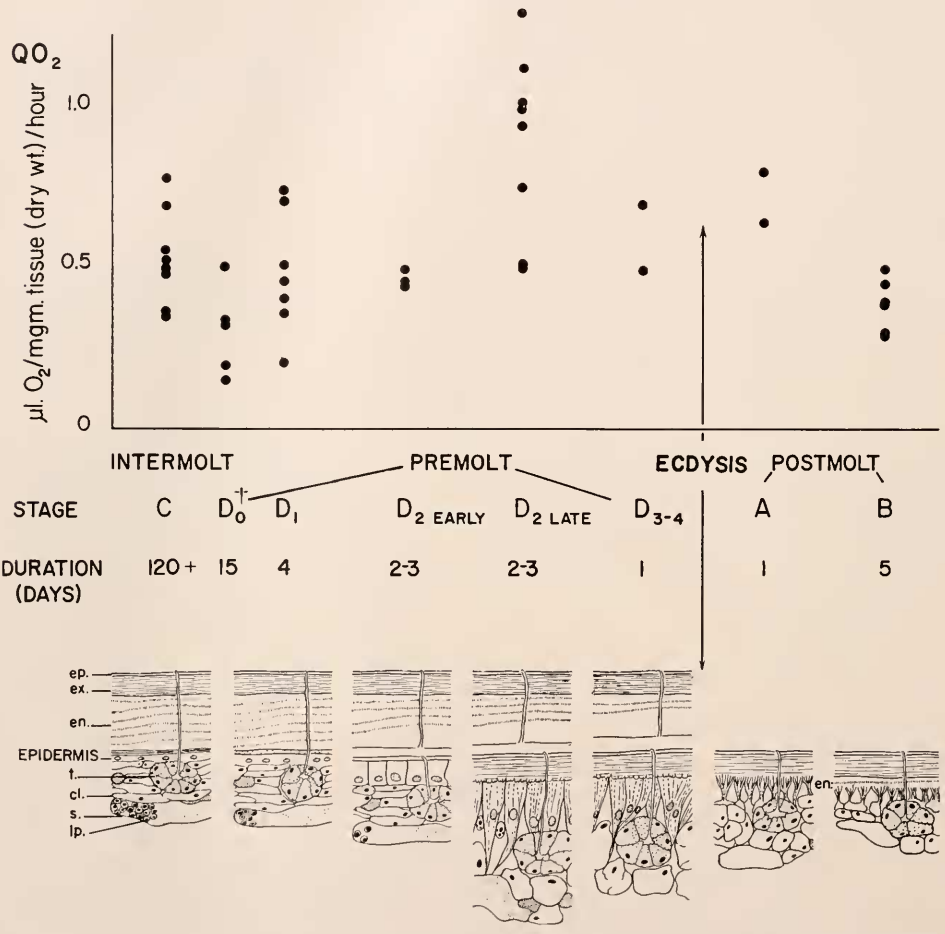


FIGURE 9. The $Q_{O_2}$ and cytology of the integumentary tissue of *Gecarcinus* at each stage of the molt cycle. In stage $D_0$, the cytology of the integumentary tissue is the same as in the intermolt period. ep = epicuticle; ex = exocuticle; en = endocuticle; t = tegumental gland; cl = cell of Leydig; s = blood sinus; lp = lipoprotein cell.

Attempts at replacing the unknown stimulating components of blood serum with Krebs' substrates (Krebs, 1950) produced only minor increases in oxygen consumption (14 to 30%).

## DISCUSSION

As can be seen in Table I, morphological evidence indicates that the first 15 days of the 30-day premolt period in *Gecarcinus lateralis* are devoted to limb

regeneration and gastrolith formation. However, it is obvious that during this first portion of the premolt period the integumentary tissues, which retain their intermolt morphology, are active in resorbing calcium from the exoskeleton and allowing its passage to the blood for storage as gastroliths in the stomach lining. Additional evidence of the catabolic activity of the integumentary tissues, preceding any change in their structure, is seen as the membranous layer of the exoskeleton is resorbed.

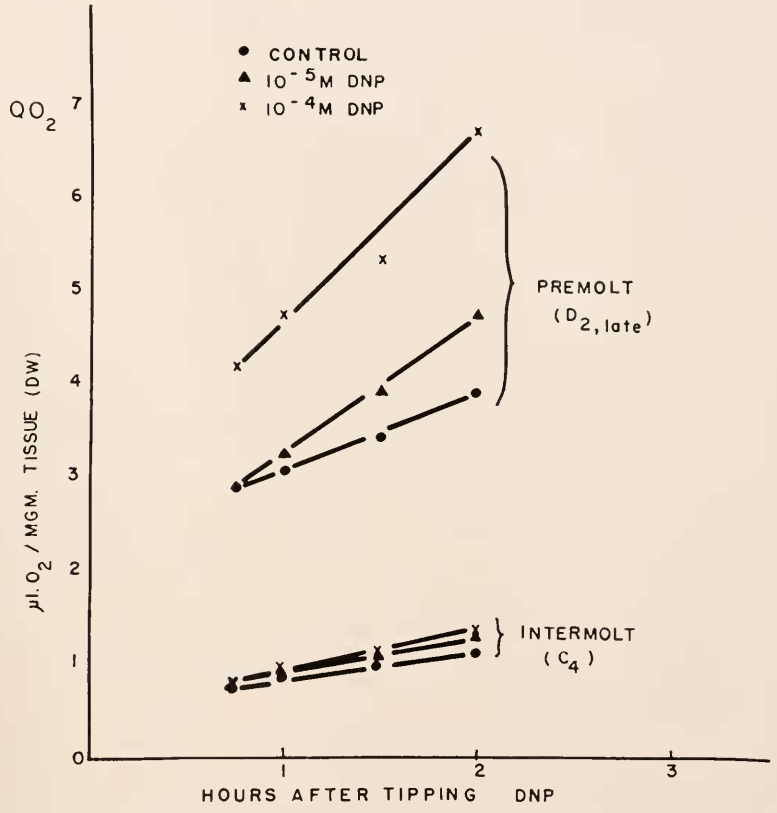


FIGURE 10. The effect of DNP on intermolt and premolt integumentary tissues. After a three-hour incubation period, DNP was tipped from the sidearm into the main vessel.

The time course of events as found in *Gecarcinus lateralis* has been fitted into the stages of Drach (1939) in Table I. Stage $D_1$, marked by the resorption of the membranous layer of the exoskeleton, occurs 11 days before ecdysis. Synthesis of epicuticle on the seventh day before ecdysis signals the beginning of Stage $D_2$. On the fourth day before ecdysis, exocuticle formation begins. This stage has been called $D_{2,\,late}$ to distinguish it from $D_{2,\,early}$ because it is during $D_{2,\,late}$ that the oxygen consumption of the integumentary tissues increases. $D_3$ and $D_4$, 1.5 days immediately preceding molt, are marked by no further synthesis of exoskeleton. There is, however, some reduction in the size of the epidermal cells. The blood of $D_3$ and $D_4$ animals has lost the characteristic blue color of crustacean

blood and assumed a pink tinge, due to astaxanthin resorbed from the old exoskeleton (Skinner and Krinsky, unpublished observations).

The increased rate of oxygen consumption of integumentary tissues in $D_{2,\,late}$ has been attributed to the rapid synthesis of exoskeleton. The 30-$\mu$-thick exocuticle, composed of approximately equal parts of chitin and protein, is synthesized in this two-day period.

The author would like to express her deep appreciation to her sponsor, Dr. John H. Welsh, for his helpful discussions during the course of this work.

## Summary

1. The morphological changes undergone during the molt cycle by the integumentary tissue of the land crab, *Gecarcinus lateralis,* have been described.

2. The time course of limb regeneration and gastrolith formation has been correlated with the morphological changes of the integumentary tissue. The period of premolt activity during which limb regeneration and gastrolith formation occurs precedes the changes in the integumentary tissues and has, therefore, been called $D_0$.

3. The oxygen consumption of the integumentary tissues has been measured at each stage of the molt cycle. It has been found to increase at the time of synthesis of the exocuticle. The effects of cyanide, dinitrophenol and added substrates on the oxygen consumption of the integumentary tissue have been studied.

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